PATENT SPECIFICATION (11) 1 549 246

1 549 246

(21) Application No. 43258/76 (22) Filed 19 Oct. 1976
(31) Convention Application No. 238 305
(32) Filed 22 Oct. 1975 in
(33) Canada (CA)
(44) Complete Specification published 25 July 1979
(51) INT CL² C07G 7/00; A61K 31/74
(52) Index at acceptance
C3H 203 220 241 242 H1
A5B 180 23X 23Y 320 32Y H

(72) Inventor JEFFREY TZE-FEI WONG

(19)

(54) BLOOD SUBSTITUTE BASED ON HAEMOGLOBIN

(71) We, HEMATECH INC., a corporation of Ontario, Canada, of Toronto-Dominion Bank Building, Toronto-Dominion Center, Toronto, Ontario, Canada, do hereby

declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to blood substitutes, and to methods for their preparation. More particularly, it relates to novel compounds and compositions which can be administered to human patients as a blood substitute by transfusion.

It is a well known and well documented fact that the demand for blood supplies for administration to patients undergoing surgery and other emergency medical procedures has increased very rapidly over the past thirty years or so. The demand often exceeds the supplies available from human donors. Even larger volumes of blood would be used if it were readily available. Effective surgery is often postponed because of shortages of blood. Medical techniques continue to become more sophisticated and successful, so that the amounts of blood required continue to increase. Extracorporeal techniques require large quantities of blood, mostly for temporary use. There is therefore a need to develop blood substitutes, and to make the most efficient use of blood supplies which are available. This need exists not only in areas where advanced medical techniques are practised, but also in underdeveloped areas of the world where expensive facilities for blood banking and blood typing are not available.

A vital function of blood in the body is the delivery of oxygen to the cells and tissues of the body, so as to maintain the functions of the various body organs. Certain compounds such as dextran, hydroxyethyl starch, polyvinylpyrrolidone and gelatin have been proposed in the past for use as blood substitutes or plasma volume expanders. However, they do not possess the required ability to deliver oxygen and yield up the oxygen to the body tissues, so that they are not useful for the management of acute haemorrhage.

Two kinds of preparation have been proposed as oxygen-carrying blood substitutes. Some perfluoro compounds such as perfluorotributylamine, perfluorodecalins and perfluorocyclic ethers can be prepared in the form of stable emulsions which have a high capacity for delivering oxygen. Whilst these compounds appear to be generally free from short term side effects on the body, other than incidences of lung lesions and thromobocytopenia, their possible long term toxicity is currently known. Also, the compounds are difficult to synthesize and purify.

The second kind of preparation is haemoglobin solution. Haemoglobin is well known to be a principal constituent of the red cells present in blood. It is a complex protein material containing molecules of iron. Its composition and structure have been extensively studied and reported in the literature. Its function in the animal body system is understood to be the transportation of oxygen to cells and tissues. Haemoglobin has the power of combining with oxygen easily and giving up the oxygen readily when the body requires it.

Use of haemoglobin solutions has the advantage, as compared with use of whole blood, that blood typing does not have to be undertaken. Such solutions therefore can be given to a patient in an emergency without taking the time to type and cross-match the blood. Blood types are understood to be determined by certain antigens present in the red cells of blood and certain natural antibodies present in the blood serum. Haemoglobin is not responsible for blood typing. Moreover, haemoglobin is a much easier material to store than whole blood, and does not deteriorate as quickly. Stocks of blood have to be discarded after a relatively short period of time. Haemoglobin can be isolated from blood and frozen

so that it can be stored for much longer periods of time. Use of haemoglobin solution instead of whole blood thus has significant advantages, and tends to alleviate problems of lack of supply of whole blood, particularly lack of supply of blood of specific types.

However, haemoglobin is rapidly excreted from the kidney as urine from the sick patient. Frequent massive transfusions of haemoglobin solution must therefore be employed, and the high rate of excretion poses a potential hazard to patients with pre-existing renal disease. It has been reported that the half-disappearance time from the circulation, of haemoglobin administered as solution by transfusion, is only 1.5 hours in monkeys.

The present invention provides a product having oxygen-transporting capability, which may be used in a blood substitute or blood extender composition, and which is the high molecular weight water-soluble product obtained by covalently coupling haemoglobin with a polysaccharide material selected from dextran, α-hydroxyethyl starch, and β-hydroxyethyl starch, which has a molecular weight of from 5,000 to 2,000,000.

Also, according to the present invention, there is provided a process of preparing a blood substitute or blood extender composition suitable for administration to an animal or human patient, which comprises coupling haemoglobin with a polysaccharide material selected from dextran, α-hydroxyethyl starch and β-hydroxyethyl starch, of a molecular weight from 5,000 to 2,000,000.

The preferred products of the invention are macromolecular products having molecular weight of at least 70,000 and having the formula (PS)—X—(HB) where PS is the polysaccharide, HB is a haemoglobin and X is a covalently bonded bridging group.

The products may be formulated as blood substitute or blood extender compositions in any suitable manner. Such compositions preferably comprise an aqueous solution of the product. The solution may be buffered in conventional manner.

The problem of rapid excretion of a haemoglobin when administered as a solution appears at least in part to be a consequence of its relatively low molecular weight and the chemical product according to the present invention has been found to have a sufficiently high molecular weight to allow its adequate retention in the body. In addition, the product according to the invention has reversible oxygen transportation capacity, allowing it to contribute to this important function of normal blood.

The preferred process for preparing the complex product according to the invention comprises the steps of first reacting the polysaccharide with a suitable chemical reagent to form a modified polysaccharide having on the polysaccharide molecule a chemical group capable of chemical interaction with groups on the haemoglobin. The polysaccharides used in the present invention, dextran and α- and β-hydroxyethyl starch, have a plurality of hydroxyl groups in the molecule. Thus, a reagent is chosen to form the modified polysaccharide which is capable of reacting with the hydroxyl groups without of course deleteriously affecting the polysaccharide in other respects. Such reagents are well known in the art, and include those having chemical groups such as carboxylic acid anhydride, acyl halide, substituted alkyl halide and sulfate, cyanogen bromide, periodate, isocyanate and epichlorohydrin.

These reagents used for preparing the modified polysaccharide should, in addition to the above-mentioned chemical group for reacting with hydroxyl on the polysaccharide, be capable of putting, onto the polysaccharide, groups capable of subsequent reaction with a haemoglobin, or with some bridging compound capable of subsequent reaction with haemoglobin. As previously noted, haemoglobin is a complex protein material. Thus it has polypeptide chains containing the polypeptide linkage — CHR — CO — NH — R'CH — derived from amino acid units. A fairly large variety of different aminoacids are involved in the haemoglobin chains, and these aminoacids provide chemical side groupings on the haemoglobin protein molecules which are available for chemical reaction with the modified polysaccharide.

Among such available groups on the haemoglobin are the following:

amino
phenolic hydroxyl
sulfhydryl
thiomethyl
imidazo
carboxyl
guanidine

Thus there is preferably used in the present invention a modified polysaccharide containing at least one chemical group capable of reacting with at least one of the aforementioned available groups on the haemoglobin molecule. Such suitable chemical groups on the modified polysaccharide are as follows:—

acylating groups which react with the amino groups on the protein, for example acid anhydride groups, N-acylimidazole groups, acid azide groups, N-carboxy anhydride groups, diketene groups, dialkyl pyrocarbonate groups, imidoseter groups, O-alkyl isourea groups, S-alkyl isourea groups, sulfonyl halide groups, sulfonate ester groups, and carbodiimide-activated carboxyl groups. All of the above groups are known to react with amino groups on proteins to form covalent bonds, involving acyl or similar linkages;

alkylating groups which react with sulf-

hydryl (mercapto), thiomethyl, imidazo or amino groups on the protein, such as halocarboxyl groups, maleimide groups, activated vinyl groups, ethylenimine groups, aryl halide groups, 2 - hydroxy - 5 - nitro - benzyl bromide groups; and aliphatic aldehyde and ketone groups together with reducing agents, reacting with the amino group of the protein;

ester- and amide-forming groups which react with a carboxyl group of the protein, such as diazocarboxylate groups, and carbodiimide and amine groups together;

disulfide-forming groups which react with the sulfhydryl groups on the protein, such as 5,5'-dithiobis(2-nitrobenzoate) groups and alkylmercaptan groups (which react with the sulfhydryl groups of the protein in the presence of oxidizing agents such as iodine);

dicarbonyl groups, such as cyclohexandione groups, and other 1,2-diketone groups, which react with the guanidino moieties of protein;

diazo groups, which react with phenolic groups on the protein molecular; and

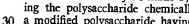
reactive groups obtained by reaction of cyanogen bromide with the polysaccharide, which react with amino groups on the protein.

Thus, in summary, the complex according to the invention may be made by first modifying the polysaccharide chemically to produce a modified polysaccharide having at least one chemical group thereon which is capable of reacting with an available chemical group on the haemoglobin protein, and then reacting together the modified polysaccharide and the haemoglobin to form a covalently bonded complex thereof utilizing the chemical group reacted onto the modified polysaccharide. Reactions of the various groups referred to above which can be put on the polysaccharide with proteins are known in the art—see for example "Chemical Modification of Proteins" by Means & Feeney, published by Holden Day, 1971, and "Advances in Carbohydrate Chemistry and Biochemistry", Vol. 29, edited by R. S. Tipson and D. Horten, published by Academic Press, with chapter by Kennedy on polysaccharide derivatives.

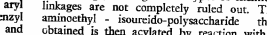
It is preferred according to the invention to react the polysaccharide to produce a modified polysaccharide having groups which will react with the sulfhydryl groups of the haemoglobin. Particularly preferred groups for this purpose are the halocarboxylate groups.

Specific examples of preferred synthetic methods for preparing the complex according to the invention are as follows:

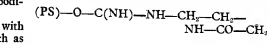
Method I: The polysaccharide (PS) is initially reacted with cyanogen bromide (CNBr) to form an activated intermediate which is reacted with diaminoethane to form an aminoethyl - isoureido - polysaccaride of the formula



The linkage between the ethyl group and the polysaccharide is most likely an isourea-type linkage, although other types of chemical linkages are not completely ruled out. The aminoethyl - isoureido-polysaccharide thus obtained is then acylated by reaction with a haloacetyl halide ($YCH_2—COZ$, Y and Z each being a halogen atom), e.g. bromoacetyl bromide, to form a haloacetyl - aminoethyl-isoureido - polysaccharide of the formula

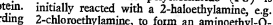


This, in turn, is reacted with the sulfhydryl groups of haemoglobin (HB) to form a haemoglobin - S - acetylaminoethyl - isoureido-polysaccharide of the formula

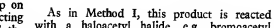


The polysaccharide is preferably dextran and Y is preferably bromine.

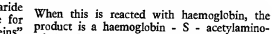
Method II: The polysaccharide (PS) is initially reacted with a 2-haloethylamine, e.g. 2-chloroethylamine, to form an aminoethyl-O-polysaccharide of the formula



As in Method I, this product is reacted with a haloacetyl halide, e.g. bromoacetyl bromide. A haloacetylaminoethyl - O-polysaccharide is obtained, having the formula



When this is reacted with haemoglobin, the product is a haemoglobin - S - acetylaminoethyl - O - polysaccharide of the formula

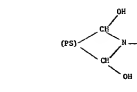


The polysaccharide is preferably dextran and Y is preferably bromine.

Method III: A polysaccharide of the formula

is reacted with sodium periodate to form a dialdehyde of the formula

Reaction between the dialdehyde and the amino groups of haemoglobin (HB) yields a haemoglobin-N-polysaccharide of the formula



The polysaccharide is preferably dextran. By proper adjustment of the conditions under which the modified polysaccharide is reacted with the haemoglobin, a yield of over 90% of coupled complex product can be obtained, rendering separation of the product from residual reactants unnecessary. For example, where the modified polysaccharide is a N - bromoacetyl - aminoethylisoureido-dextran (Br-dextran) prepared as described above, the concentrations of the haemoglobin and Br-dextran in the coupling reactant solution, and the reaction time, can be adjusted to give over 90% yields of coupled products. Too high a concentration of reactants leads to gelation of the reactant solution and formation of a cross-linked product of excessively high molecular weight which is usually undesirable. It is preferred to use a molar ratio of Br-dextran to haemoglobin close to one, or less than one in the case where a dextran of high molecular weight is used. Formation of cross-linked product may also be inhibited by lowering the pH to stop the alkylation reaction or by adding mercaptoethanol or cysteine to react with Br-dextran in competition with the haemoglobin sulfhydryls.

As previously noted, the polysaccharide used according to the present invention has a molecular weight of from 5,000 to 2,000,000. The preferred molecular weight range, especially in the case of dextran, is from 5,000 to 200,000, and is most preferably from 20,000 to 70,000. Within such molecular weight ranges, coupling with haemoglobin takes place readily, and the reaction solutions have suitable viscosities for ease of handling. In addition, dextrans of molecular weight below 90,000 are known to be substantially non-allergenic, and are therefore desirable for use in the present invention.

The coupled product of the polysaccharide and haemoglobin may be a one-to-one coupling, or there may be several, e.g. up to 9, molecules of haemoglobin coupled to one molecule of polysaccharide. This can be controlled by the relative amounts of reactants in the coupling reaction, and control of other reaction conditions such as time, temperature and pH. The preferred products of the present invention have molecular weights of at least 70,000. The most preferred molecular weights for the product are from 85,000 0to 135,000.

The haemoglobin-polysaccharide complex may be recovered in a physiologically acceptable carrier ready for administration to a patient. The reaction medium in which the complex is formed may constitute the carrier, provided it is physiologically acceptable.

The following Examples illustrate the invention.

Example 1.

Preparation of Dextran-Haemoglobin Complex.

0.3 gm of cyanogen bromide is dissolved in 3 ml of acetonitre and added to 100 ml of 2% w/v dextran solution (mol. wt. 200,000). The pH is maintained at 10.8 with 1 M NaOH for 5 minutes. 2 ml of ethylenediamine are then added. The pH is adjusted to 9.5 with concentrated HCl, and the reaction mixture is left overnight.

The mixture is dialysed thoroughly against distilled water and freeze-dried. The freeze-dried aminated dextran can be stored for a long time.

All the activated dextran recovered (1.6—1.7 gm) is dissolved in 50 ml 0.1 phosphate buffer, pH 7.0, and 2 ml of bromoacetyl bromide is very slowly added, with vigorous stirring, over a period of 2 hrs. The pH is constantly maintained at 7.0 with the addition of 1M NaOH. When the reaction is over, the mixture is dialysed thoroughly against distilled water and then freeze-dried. 1.4 gm of brominated dextran, or Br-dextran is recovered.

1 gm of Br-dextran is added to 30 ml of a 2—3% w/v solution of human haemoglobin in 0.1M sodium bicarbonate buffer, pH 9.5, and the reaction is allowed to go overnight.

Dextran-haemoglobin and free haemoglobin are separated from each other on a Sephadex (registered Trade Mark) G-200 column. Yield of dextran-haemoglobin is 70—80% of the total haemoglobin added.

Example 2.

Renal Excretion of Haemoglobin and Dextran-Haemoglobin by Rats.

To test the effectiveness of the complex according to the present invention as a blood substitute, 3 ml of a 2% w/v dextran-haemoglobin complex solution, prepared according to Example 1, was infused into a Wistar rat, and the amount of dextran-haemoglobin excreted by the animal was estimated by washing the bladder with a continuous stream of physiological saline, and measuring the amount of dissolved haemoglobin in the wash as a function of time. An exactly similar control experiment was run, except using 3 ml of a 2% w/v haemoglobin solution. In both instances, the haemoglobin content was determined spectrophotometrically in terms of optical density at 415 nm.

The results are shown graphically on the attached Figure. This is a graphical representation of optical density plotted against

time, for the respective experiments. It will be seen that the rate of excretion of the haemoglobin is much greater than the rate of excretion of the dextran-haemoglobin complex.

This experiment demonstrates that dextran-haemoglobin is potentially a much more useful blood substitute than free haemoglobin with respect to its vastly improved retention by the animal against renal excretion.

Example 3.

2 g of dextran (weight average molecular weight 110,000) was dissolved in 75 ml distilled water, the pH was adjusted to 10.8 with 2M NaOH, and to this 0.3 g cyanogen bromide dissolved in 3 ml of acetonitrile was added with stirring at room temperature. The pH was maintained at 10.8 for 5 minutes by addition of 2M NaOH. The pH was then adjusted to about 2.0—2.5 with concentrated HCl and the solution was stirred for another minute. 3 ml of ethylenediamine were added along with additional HCl to prevent the pH from exceeding 9.5; the final pH was adjusted to 9.5. The solution was stirred overnight at 4°C. The aminated dextran formed was dialyzed in a Bio-Fiber 50 beaker (Bio-Rad Laboratories) against deionized water until no free amine could be detected in the dialysate by ninhydrin. The dialyzed solution was lyophilized to give about 1.6 g of dried aminated dextran. This was dissolved in 50 ml 0.1M phosphate buffer, pH 7.0, and 3 ml of bromoacetyl bromide was added through a pasteur pipette with a finely drawn capillary tip over a period of 60 minutes. Throughout the solution was stirred vigorously in an ice-water bath, and maintained at pH 6.6 to 6.8 by means of a pH-stat with the addition of 2M NaOH solution during the addition of bromoacetyl bromide. Afterwards the solution was dialyzed against deionized water until no free bromine could be detected in the dialysate by silver nitrate. About 1.5 g of Br-dextran was obtained upon lyophilization. The experiment was repeated using other dextrans of average molecular weight 200,000; 70,000; 40,000; and 20,000. The bromine content of the various Br-dextrans synthesized, determined on the basis of elemental analysis, was in the range 9—11 glucose residues per bromine atom.

The Br-dextrans so formed were coupled with haemoglobin, by dissolving a specified amount in 6 ml haemoglobin solution (containing 2.5, 5 or 10% w/v haemoglobin in 0.1M sodium bicarbonate, pH 9.5). The coupling was allowed to proceed with constant mixing at 4°C. Yields of coupled products were determined by eluting the reaction mixture on a Sephadex column with 0.05 M phosphate buffer, pH 7.5. Haemoglobin content was determined by absorbance at specified wavelengths. Results are given in Table 1.

TABLE I

Dextran Wt.	% Br-Dextran in Reactant Soln (w/v)	% Haemoglobin in Reactant Soln (w/v)	Molar Ratio of Dextran/ Haemoglobin	Reaction Time (Hrs.)	Viscosity (Centi-Stokes)	% Yield of Coupled Product
200,000	3.33	5.0	0.21	24	35.19	96
200,000	3.33	5.0	0.21	48	gelled	
200,000	3.33	2.5	0.43	24	9.86	97
200,000	1.66	5.0	0.11	24	7.56	92
200,000	1.66	5.0	0.11	48	8.47	96
110,000	3.33	5.0	0.39	24	20.94	95
110,000	3.33	5.0	0.39	48	45.29	97
110,000	3.33	5.0	0.39	72	gelled	
110,000	3.33	2.5	0.78	24	7.43	97
110,000	1.66	5.0	0.19	24	6.43	85
110,000	1.66	5.0	0.19	48	7.39	93
110,000	1.66	5.0	0.19	72	8.07	95
70,000	3.33	5.0	0.61	24	19.40	99
70,000	3.33	2.5	1.22	24	6.72	98
70,000	1.66	5.0	0.31	24	5.81	87
70,000	1.66	5.0	0.31	48	6.49	94
70,000	1.66	5.0	0.31	72	6.90	96
40,000	3.33	10.0	0.54	24	15.37	83
40,000	3.33	10.0	0.54	48	20.10	90
40,000	3.33	10.0	0.54	72	22.65	94
40,000	3.33	5.0	1.07	24	6.32	96
40,000	3.33	2.5	2.15	24	4.14	99
40,000	1.66	5.0	0.54	72	4.29	92

TABLE I (Continued)

Dextran Wt.	% Br-Dextran in Reactant Soln (w/v)	% Haemoglobin In Reactant Soln (w/v)	Molar Ratio of Dextran/ Haemoglobin	Reaction Time (Hrs.)	Viscosity (Centi-Stokes)	% Yield of Coupled Product
20,000	33.3	10.0	1.07	48	8.91	97
20,000	3.33	10.0	1.07	72	10.24	98
20,000	1.66	5.0	1.07	72	3.07	94

These results show that with each dextran over 90% yields of coupled product can be obtained by suitable choice of experimental conditions.

Example 4.

Preparation of Dextran-Haemoglobin Complex by Method II.

One gm of dextran (mol. wt. 40,000) was thoroughly mixed with 1 ml of β-chloroethylamine, which was obtained as the upper phase from an addition of concentrated NaOH to β-chloroethylamine hydrochloride. The mixture was further mixed with 0.4 ml of concentrated NaOH, placed in a capped tube and autoclaved at 120°C for 1 hour. Then 1 ml of β-chloroethylamine and 0.4 ml of concentrated NaOH were added, and the mixture again autoclaved at 120° for 1 hour; this was repeated yet another time. After cooling, the mixture was thoroughly dialysed against distilled water and placed finally in 11 ml of 0.1 M phosphate buffer, pH 6.8.

This solution of aminoethyl-O-dextran was acylated with the slow addition of 0.5 ml of bromoacetyl bromide over a period of about 1 hour. It was thoroughly dialyzed against distilled water and freeze-dried.

0.1 gm of the freeze dried bromoacetyl-aminoethyl-O-dextran was added to 1.7 ml of 5% w/v human haemoglobin in 0.1 M sodium bicarbonate buffer, pH 9.5, and held at 4°C for 48 hours. Chromatography of Sephadex indicated that over 90% of the haemoglobin was coupled in the form of dextran-haemoglobin.

Example 5.

Preparation of Dextran-Haemoglobin Complex by Method III.

One millilitre of a 12% w/v aqueous solution of sodium periodate was added to 10 ml of a 10% w/v aqueous solution of dextran, and the mixture was left overnight in the dark at 4°C. A 3% w/v solution of sodium bisulfite was added until the mixture turned brown and then, once again, colourless. The mixture was dialyzed against distilled water to yield the dextran-dialdehyde solution. It was then added to 2 volumes of 3% w/v stroma-free haemoglobin in 0.3 M sodium bicarbonate buffer, pH 9.5. Coupling of haemoglobin to dextran was allowed to proceed overnight at 4°C. The dextran-haemoglobin complex formed was separated from uncoupled haemoglobin by means of chromatography on a Sephadex G-200 column. About 60% yield of coupled product was obtained.

Example 6.

Renal Excretion of Haemoglobin and Dextran-Haemoglobin by Rabbits.

Male rabbits, of body weight 3.3—3.5 kg, were anesthetized with 0.1 g of sodium pentothal. A solution of haemoglobin or dextran-haemoglobin (molecular weight of dextran: 200,000—275,000) according to the invention, in a standard kidney dialyzing buffer (according to Rabiner et al, 1967, J. Exp. Med. *126*, 1127—1142), containing 20μ Ci (microCuries) of [^{3}H] methoxy-inulin, was infused into each animal through the marginal ear vein at 1.1 ml/minute. After the solution had been infused, infusion was continued at the same rate with buffer to maintain urinary output. At intervals, the content of the bladder was washed out with three 5-ml portions of 0.9% w/v saline with the use of a Foley no. catheter (3 ml) and, after centrifugation at 3000 × g for 10 minutes to remove any sedimentable material, the dissolved haemoglobin or dextran-haemoglobin in the combined washes was determined on the basis of absorbance at 576 nm. The [^{3}H] insulin content in the combined washes was measured by scintillation counting with correction for quenching by haemoglobin; an external radiation standard in the Nuclear Chicago Mark II counter was used to determine quenching. Plasma concentration of a haemoglobin or dextran-haemoglobin was determined at various times by withdrawing blood samples from the carotid artery and making absorbance measurements on the samples at 576 nm after sedimenting the erythrocytes.

Tests were conducted using 50 ml or 30 ml samples of 1% w/v haemoglobin, or dextran-haemoglobin containing 1% w/v equivalent of haemoglobin.

It was found that the dextran-haemoglobin, specifically that produced by the method of Examples 1 and 3, was excreted through the kidneys and removed from circulation at a greatly reduced rate compared to free haemoglobin even though renal function in the animals infused with dextran-haemoglobin, as indicated by inulin excretion, was unimpaired. Furthermore, since it was repeatedly observed with different animals and at different dosages of infusion, this dissimilar physiological behaviour of dextran-haemoglobin and free haemoglobin was due to the different nature of the substances, not to some chance variation in, for example, the blood haptoglobin level of the experimental animals.

The oxygen-binding characteristics of products according to the present invention are determined by the method of Benesch *et al*, (1965) Anal. Biochem. *11*, 81—87. It is found that, as compared with hemoglobin, the products according to the present invention tend to show a somewhat greater affinity for oxygen, but retain the essential oxygen-transporting and releasing capability of haemoglobin. As measured by the half-saturation oxygen tension, the dextran-haemoglobin complex prepared by method I described above shows approximately 2.5-fold greater affinity for oxygen compared to free haemoglobin. The oxygen affinity of the complex can be varied by suitable chemical treatment of the haemoglobin, before or after coupling with the polysaccharide, for example by reacting it with pyridoxal phosphate and reducing with sodium borohydride.

WHAT WE CLAIM IS:—

1. A high molecular weight water-soluble product having oxygen-transporting capability, which may be used in a blood substitute or blood extender composition and which is obtainer by covalently coupled haemoglobin and a polysaccharide which has a molecular weight of from 5,000 to 2,000,000 and which is selected from dextran, α-hydroxyethyl starch and β-hydroxyethyl starch.

2. A product according to claim 1 having a molecular weight of at least 70,000 and the general formula

(PS)—X—(HB)

wherein PS is the polysaccharide, HB is haemoglobin and X is a covalently bonded chemical bridging group.

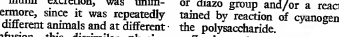
3. A product according to claim 1 or claim 2 wherein the polysaccharide has a molecular weight of from 5,000 to 200,000.

4. A product according to claim 3 wherein the polysaccharide has a molecular weight of from 20,000 to 70,000.

5. A product according to any preceding claim wherein the polysaccharide is modified and contains groups capable of reaction with amino, phenolic hydroxyl, sulfhydryl, thiomethyl, imidazo or carboxyl side groups on the haemoglobin.

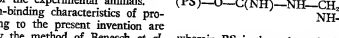
6. A product according to claim 5 wherein the polysaccharide is modified to contain at least one acylating, alkylating, ester-forming, amide-forming, disulfide-forming, dicarbonyl or diazo group and/or a reactive group obtained by reaction of cyanogen bromide with the polysaccharide.

7. A product according to any preceding claim which is the product of reacting haemoglobin with a bromoacetyl - aminoethyl - isoureido - polysaccharide of the formula



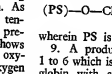
wherein PS is the polysaccharide.

8. A product according to any of claims 1 to 6 which is the product of reacting haemoglobin with a bromoacetyl - aminoethyl - O-polysaccharide of the formula



wherein PS is the polysaccharide.

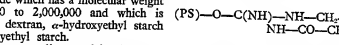
9. A product according to any of claims 1 to 6 which is the product of reacting haemoglobin with a dialdehyde polysaccharide of the formula



wherein PS is the polysaccharide.

10. A product according to any preceding claim wherein the polysaccharide is dextran.

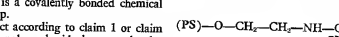
11. A haemoglobin - S - acetylaminoethyl-isoureido - polysaccharide of the formula



wherein PS is the polysaccharide and HB is haemoglobin.

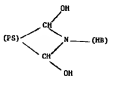
12. A product according to claim 11 wherein the polysaccharide is dextran.

13. A haemoglobin - S - acetylaminoethyl-O - polysaccharide of the formula



14. A product according to claim 13 wherein the polysaccharide is dextran.

15. A haemoglobin - N - polysaccharide of the formula



wherein PS is the polysaccharide and HB is haemoglobin.

16. A product according to claim 15 wherein the polysaccharide is dextran.

17. A product according to claim 1 substantially as described in any of Examples 1, 3, 4 and 5.

18. A process of preparing a product which may be used as a blood substitute or blood extender, which comprises covalently coupling haemoglobin with a polysaccharide having a molecular weight of from 5,000 to 2,000,000 and selected from dextran, α-hydroxyethyl starch and β-hydroxyethyl starch, to form a water-soluble covalently-coupled complex thereof.

19. A process according to claim 18 which comprises

(a) chemically modifying the polysaccharide to introduce into the polysaccharide groups capable of reaction with haemoglobin and selected from acylating, alkylating, ester-forming, amide-forming and disulfide-forming groups; and

(b) reacting the modified polysacharide with haemoglobin.

20. A process according to claim 18 which comprises (a) reacting the polysaccharide with cyanogen bromide; (b) reacting the product of step (a) with ethylenediamine to form an aminoethyl - isoureido - polysaccharide of the formula

$$(PS)—O—C(NH)—NH—CH_2—CH_2—NH_2$$

wherein PS is the polysaccharide;

(c) acylating the aminoethyl - isoureido-polysaccharide with a haloacetyl halide of the formula

$$YCH_2—CO—Z$$

wherein Y and Z are the same or different halogen atoms, to form a haloacetyl - aminoethyl - isoureido - polysaccharide of the formula

$$(PS)—O—C(NH)—NH—CH_2—CH_2—NH—CO—CH_2Y$$

wherein PS and Y are as defined above; and

(d) reacting the haloacetyl - aminoethyl-isoureido - polysaccharide with haemoglobin to form a product according to claim 11.

21. A process according to claim 18 which comprises

(a) reacting the polysaccharide with a 2-haloethylamine to form an aminoethyl - O-polysaccharide of the formula

$$(PS)—O—CH_2—CH_2—NH_2$$

wherein PS is the polysaccharide;

(b) reacting the aminoethyl - O - polysaccharide with a haloacetyl halide as defined in claim 20 to form a haloacetyl - aminoethyl-O - polysaccharide of the formula

$$(PS)—O—CH_2—CH_2—NH—CO—CH_2Y$$

wherein PS is the polysaccharide and Y is halogen atom; and

(c) reacting the haloacetyl - aminoethyl-O - polysaccharide with haemoglobin to form a product according to claim 13.

22. A process according to claim 18 substantially as described in any of Examples 1, 3, 4 and 5.

23. The product of a process according to any of claims 18 to 22.

24. A blood substitute or blood extender composition which comprises an aqueous solution of a product according to any of claims 1 to 17 and 23.

25. A composition according to claim 24 substantially as described in Example 2 or Example 6.

For the Applicants:
GILL, JENNINGS & EVERY,
Chartered Patent Agents,
53/64 Chancery Lane, London, WC2A 1HN.

Printed for Her Majesty's Stationery Office by the Courier Press, Leamington Spa, 1979.
Published by the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

1549246 COMPLETE SPECIFICATION
1 SHEET This drawing is a reproduction of the Original on a reduced scale